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The Role of Carbohydrate in Determining the Immunochemical Properties of the Hemagglutinin of Influenza A Virus

By

A. K. Gitelman, V. A. Berezin, and I. G. Kharitonenkov

D. I. Ivanovsky Institute of Virology, U.S.S.R. Academy of Medical Sciences, Moscow, U.S.S.R.

With 6 Figures

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Summary

Most of the carbohydrate was removed from influenza virus MRC II (H3N2) and its purified hemagglutinin (HA) on treatment with glycosidases, including α-mannosidase, β-N-acetylglucosaminidase, β-galactosidase and α-fucosidase. The release of 50 per cent of the carbohydrate from intact virus particles significantly affected hemagglutinating activity. The ability of untreated and glycosidase-treated virus to inhibit the binding of antibodies directed against the hemagglutinin was almost indistinguishable by competitive radioimmunoassay (RIA). Up to 60 per cent of the carbohydrate from the purified HA of influenza virus could be removed. The antigenicity of glycosidase treated HA molecules decreased 8-fold compared to intact HAs as measured by competitive RIA. In addition, glycosidase digestion of ^{125}I-labeled HA resulted in a decrease in its reactivity in direct RIA.

We conclude that the carbohydrate portion of the HA of influenza virus is not of major importance in defining the antigenicity of HA.

Introduction

Hemagglutinin (HA) is the major surface antigen of influenza virus. Antibodies that neutralize virus infectivity are directed against this glycoprotein, and recurrent influenza epidemics are associated with changes in its antigenic structure (21, 27, 39, 51). The HA accounts for 25 to 35 per cent of the weight of virus particles.

HA is composed of two different glycoprotein subunits which may be present in uncleaved form (HA, mol. wt. 75,000) or in cleaved form. These subunits are called heavy (HA 1) (mol. wt. of about 50,000) and light (HA2) (mol. wt. of

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about 25,000) chains and are held together by covalent disulfide bonds (26, 50). The HA molecule contains about 25 per cent carbohydrate by weight. The carbohydrate moiety of HA comprises two distinct types of oligosaccharide side chains. The predominant oligosaccharide chain, type I, has a mol. weight of about 2800 and contains N-acetylglucosamine, mannose, galactose, and fucose. The smaller oligosaccharide side chains, type II, mol. weight of about 1600 to 2200 contains high amounts of mannose in addition to N-acetylglucosamine (49, 40, 7, 33). The carbohydrate moieties are linked to polypeptides by N-glycosidic linkages between N-acetylglucosamine and asparagine (19). There are variations in carbohydrate content and in the ratios of the constituent sugars which may be due to conditions of virus growth or to the virus strain used (7, 15, 34). These differences are probably due to the nature of the glycosyl transferases present in different host cells (10, 36, 41).

However, the role of the carbohydrate components of influenza viral glycoproteins in the expression of their biological activities is not clear. Thus, virus particles were produced with unglycosylated or partially glycosylated viral glycoproteins when inhibitors of glycosylation were present during synthesis of the virus. Such particles lacked or possessed significantly reduced hemagglutinating activity (41, 22, 18, 32). On the other hand, the removal of some sugars from the carbohydrate of intact virus particles and their isolated HAs did not significantly affect hemagglutinating activity (8).

The role of carbohydrates in determining the immunological properties of viral glycoproteins has been studied mostly with oncoviruses. The removal of most of the carbohydrate from the envelope glycoprotein gp71 of Friend Murine Leukemia Virus and gp85 of avian sarcoma virus (B77) did not destroy their antigenicity (5, 37, 45). On the other hand, the removal of carbohydrates from gp85 of avian myeloblastosis virus eliminated its antigenic activity (45). The role of carbohydrate in determining the immunological specificity of influenza virus glycoproteins is still to be resolved (7, 33, 35). In this report, we have investigated the antigenic properties of influenza virus hemagglutinin after partial removal of carbohydrates.

Materials and Methods

Viruses

The influenza A virus recombinant MRC-II, which is antigenically identical to A/Port Chalmers/1/73 (H3N2), was used. The virus was grown in embryonated eggs and purified as described elsewhere (20).

Virus Assay

Virus yields were assayed by hemagglutination titrations as previously described (9).

Neuraminidase Assay

Released N-acetylneuraminic acid was determined by the method of AMINOFF (1) as recommended by the WHO procedure (2). The final NAse activity was expressed in terms of specific NAse activity.

Protein Assay

Protein was estimated by the method of LOWRY *et al.* (30) using BSA (Sigma) as standard.

Isolation of the Hemagglutinin

Bromelain-released influenza HA was obtained by treatment of purified virus preparations with bromelain (Serva B) and was purified by sucrose density gradient centrifugation (6). A commercial bromelain preparation was additionally purified by the method of LYNN (31).

The purified bromelain preparation was obtained by the courtesy of Kolesnikov, V. V. No residual neuraminidase activity was detected in the bromelain-released hemagglutinin. The isolated hemagglutinin was analyzed by SDS-polyacrylamide gel electrophoresis under nonreducing conditions and under reducing conditions for HA_1 and HA_2 polypeptides to confirm the absence of any contaminating proteins (25).

Antisera

Hyperimmune antisera to purified HA was obtained by inoculating 150 μg of antigen in complete Freund's adjuvant (CFA, Difco Laboratories, Detroit, Mich.) into lymph nodes of adult rabbits.

The same antigen dose was given intramuscularly and intravenously three weeks later, and the animals were bled after a further 7 days. The antiserum was heat-inactivated at 56° C for 30 minutes before use. The pooled serum was examined for virus-specific antibodies by hemagglutinin-inhibition (12), immuno-double diffusion (38) and immunoprecipitation (42, 4).

Iodination of the Viral Surface Glycoproteins

The surface viral glycoproteins were labeled with ^{125}I by lactoperoxidase-catalyzed radioiodination as described by STANLEY and HASLAM (44). Purified virus (500 μg) was suspended in 50 μl of phosphate-buffered saline (pH 7.2) containing 20 μg (1.4 U) of lactoperoxidase (Sigma). ^{125}I (200 μCi) was added followed by 10 μl of 0.03 per cent H_2O_2. The virus was mixed vigorously and incubated at 37° C for 5 minutes. The reaction was terminated by addition of 10 ml of chilled phosphate-buffered saline. ^{125}I-labeled virus was pelleted by ultracentrifugation and washed twice with phosphate-buffered saline. The specific radioactivity of the iodinated virus preparation was 1.2×10^5 cpm/μg protein. More than 50 per cent of this radioactivity was acid insoluble.

Iodination of HA

HA was iodinated by the procedure of GREENWOOD *et al.* (14). 10 μg of glycoprotein in 50 μl of 0.2 M phosphate buffer (pH 7.4) was reacted with 1 mCi of Na ^{125}I in the presence of 15 μg of chloramine T for 45 seconds. The reaction was terminated by the addition 50 μg of potassium metabisulfate in 300 μl of Na-phosphate buffer (pH 7.5). The entire mixture was put on to a Sephadex G-25 superfine column (10 by 0.5 cm) and eluted with RIA buffer (10 mM Tris HCl; 1 mM EDTA; 100 mM NaCl; 0.2 per cent Triton X 100; 0.2 per cent bovine serum albumin, pH 7.2). 0.5 ml fractions were collected and those containing labeled HA were pooled. The HA contained 1.5×10^7 cpm/μg of protein. 94 per cent of this radioactivity was precipitable with 10 per cent trichloroacetic acid and more than 40 per cent was precipitable with homologous antiserum at a dilution of 1 : 20.

Radioimmunoassay

The double antibody RIA procedure was performed as described by VLASENKOVA *et al.* (47). In the competition assay, increasing amounts of the material to be tested were added in a 100 μl volume to 10 μl of rabbit immune serum (at a dilution that precipitated 50 per cent of the precipitable iodinated antigen) with 5 per cent normal rabbit serum. Then 10 μl aliquots of labeled antigen (40,000 cpm/min; 3 ng) were added. All dilutions were made in RIA buffer.

The mixtures were incubated for 2 hours at 37° C. Then 50 μl of antiserum to rabbit globulins were added and incubated for 1 hour at 37° C, and then overnight at 4° C. Immunoprecipitates were washed twice with RIA buffer and counted in a Packard Autogamma counter. The percentage inhibition for each concentration of competing antigen was calculated.

Briefly, in the direct assay, dilutions of antiserum with 5 per cent normal rabbit serum were reacted with iodinated HA (50,000 cpm/min, 3 ng). The procedure described for the competition method was then followed to complete the assay procedure.

Preparation of Glycosidase Enzymes

α-D-mannosidase was prepared from bromelain by the method of LI and LEE (29). β-D-galactosidase and β-D-N-acetylglucosaminidase were purified from Sweet Almond Emulsin by the method of LEE (28). α-L-fucosidase was prepared from calf kidney as described previously (48). These enzymes were purified and assayed as described elsewhere (3). Glycosidase activities were assayed by the use of the appropriate p-nitrophenylglycosides as substrates with a unit (U) of enzyme activity defined as the release of 1 μmole of p-nitrophenol per minute at 37° C.

Specific activities of 134 U of α-mannosidase, 128 U of β-galactosidase, 109 U of β-N-acetylglycosidase, 54 U of α-fucosidase per mg of protein were obtained for the glycosidase preparations. No proteolytic activity was detected by the casein substrate assay.

Glycosidase Treatment of Virus and Isolated Hemagglutinin for Competition RIA

Virus and hemagglutinin preparations, at concentrations of 2 mg/ml and 1 mg/ml respectively, were dialyzed exhaustively against 0.05 M sodium phosphate buffer (pH 7.2), 0.85 per cent NaCl and 0.01 per cent NaN_3, prepared in tridistilled water to remove free sugars. The first buffer was then replaced by 0.05 M phosphate-citrate buffer (pH 5.75) and the glycosidase enzyme mixture containing 55 U of α-mannosidase, 32 U of β-galactosidase, 60 U β-N-acetylglucosaminidase and 27 U of α-fucosidase per 1 mg of viral protein was added and incubated at 37° C for 60 hours. Virus and hemagglutinin treated in the same way but without glycosidase (control sample), and untreated preparations of virus and hemagglutinin (initial sample) were used as controls. The reaction was terminated by pelleting the samples containing virus at $100{,}000 \times g$ for 20 minutes. The percentage of the removed sugars from the virus and its purified hemagglutinin was determined by using the ferricyanide assay technique (see below).

After digestion, samples were removed for analysis by SDS-polyacrylamide gel electrophoresis and by competition RIA.

Glycosidase Treatment of ^{125}I-Labeled Hemagglutinin for Direct RIA

^{125}I-labeled hemagglutinin (approximately 5×10^6 cpm) was digested with the glycosidase enzyme mixture as described above. Controls used were as follows: ^{125}I-labeled hemagglutinin treated in the same way without glycosidase (control sample) and untreated ^{125}I-labeled hemagglutinin (initial sample). After digestion, samples were analyzed by direct RIA.

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was done with 10 per cent resolving gels prepared by the method of LAEMMLI (25). Protein was detected by staining with amidoblack 10B (Serva) and carbohydrate was detected with the periodic acid-Schiff technique of FAIRBANKS *et al.* (11). Gels were scanned at 560 nm in a Gilford spectrophotometer equipped with a linear transport system. Gels containing iodinated proteins were fixed and cut into 1 mm slices with a gel fractionator and counted in a Packard Autogamma Counter.

Quantitation of Carbohydrate

The determination of the sugar content in untreated and glycosidase-treated virus and HA preparations was done by the ferricianide reducing test as described by KRYSTAL and GRAHAM (24). Samples of whole virus and purified HA were dialyzed to remove free carbohydrates and salts and then hydrolyzed with 2 N HCl in sealed glass vials under nitrogen at 100° C for 20 hours. Some virus samples were first treated with a glycosidase mixture, pelleted, and washed three times before hydrolysis. After hydro-

lysis, samples containing 40—50 nmole of carbohydrates, were quickly dried under a stream of nitrogen at room temperature, dissolved in 0.2 ml of tridistilled water, and then 0.4 ml methanol was added. The samples were applied to a column of AG1 × 2 (OH^- form, 200—400 mesh) ion-exchange resin Bio-Rad preequilibrated with 70 per cent methanol in order to fractionate them into monosaccharides and amino acids. The column was washed with 10 ml of 70 per cent methanol and the effluents were combined and evaporated to dryness at 40° C in a rotary Evapo-mix. The dried samples were dissolved in tridistilled water and the total sugar content was assayed by the standard ferricyanide-reducing method. The amount of sugar present in any particular sample was calculated by comparing the reducing ability of this sample with that of a standard namely α-D-mannose.

Results

Treatment of Virus and Purified HA with Glycosidase Enzymes

Lactoperoxidase-catalyzed iodination with ^{125}I allows specific labeling of the viral envelope proteins (44). This method was used to label both HA and neuraminidase (NA). ^{125}I-labeled virus was treated with the glycosidase mixture, pelleted to separate the particles from enzymes, and then analyzed by SDS-gel electrophoresis. No iodinated material was detected in the supernatant which indicated that only the carbohydrate portions of the glycoproteins were digested and that the integrity of the proteins was not affected. These results also showed there was no protease activity in the enzyme preparations used. HA_1 of treated ^{125}I virus was found to be more heterogenous in size on polyacrylamide gels than HA_1 of untreated virus (Fig. 1). Nevertheless the HA_1 band of treated virus

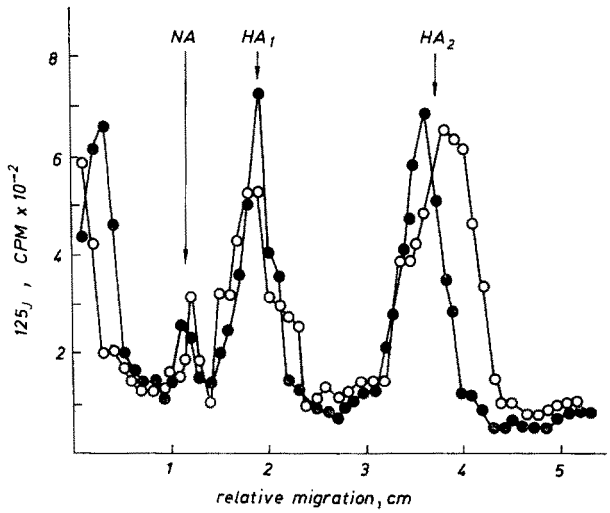

Fig. 1. SDS-polyacrylamide gel electrophoresis of ^{125}I-labeled surface glycoproteins of influenza A/MRC II after glycosidase treatment. Purified intact MRC II virus was iodinated with lactoperoxidase and treated with the glycosidase mixture as described in Materials and Methods. Untreated (•———•) and glycosidase-treated (o———o) ^{125}I-labeled viruses were electrophoresed on 10 per cent SDS-PAGE gel under reducing conditions at 3.5 mA/tube for 5 hours. Gels were sliced vertically into 1 mm discs. Each slice was counted in a Packard Autogamma Counter. Electrophoresis was from left to right

migrated to the same position as that of untreated virus. Slight differences were found in the migration rate on polyacrylamide gels of HA_1 from glycosidase treated and untreated virus (Fig. 2). HA_2 of ^{125}I-labeled (Fig. 1) and unlabeled (Fig. 2) virus treated with glycosidases was more heterogenous in size than HA_2 of untreated virus. The HA_2 band of treated virus migrated further into the gel, indicating a decrease in its size. The specificity of glycosidase for the carbohydrate moiety of HA was shown by staining for carbohydrate with periodic acid-Schiff. Most of the periodic acid-Schiff-stainable material was removed after enzyme treatment of influenza virus (Fig. 3). The intensity of staining of treated HA_1 carbohydrates was reduced considerably in comparison with that of intact HA_1. A similar, although less marked, reduction in staining with periodic acid-Schiff was observed with treated HA_2.

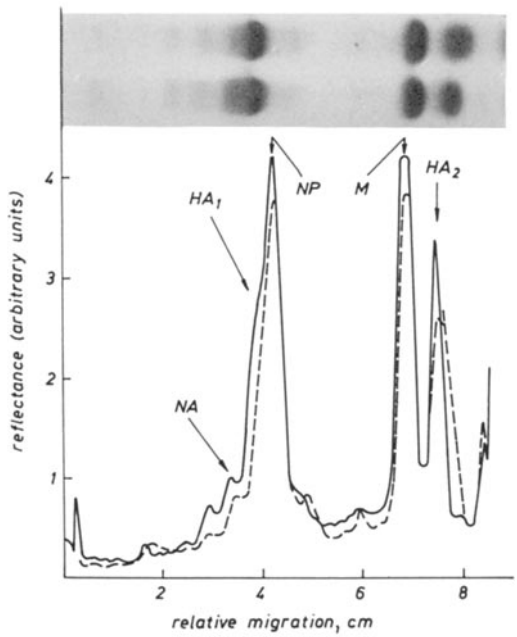

Fig. 2. Analysis of glycosidase-treated MRC II virus by electrophoresis in SDS-polyacrylamide gels. Purified virus was digested with glycosidase enzymes as described in Materials and Methods, and electrophoresed on 10 per cent SDS-PAGE gel as described in the legend of Fig. 1. Gels were stained with amido-black and scanned. The resulting curves of untreated (———) and glycosidase-treated (— — —) virus were redrawn after a base line correction. Electrophoresis was from left to right. The upper panel shows the amido-black stained gel of glycosidase-treated (top) and untreated (bottom) virus

Purified HA was treated with the glycosidase mixture and analyzed by SDS-gel elctrophoresis. The elctrophoretic mobilities of HA_1 and HA_2 were altered on treatment. Both bonds were more heterogenous than those of untreated HA (Fig. 4). The additional bands observed in glycosidase-treated HA represent glycosidase polypeptides.

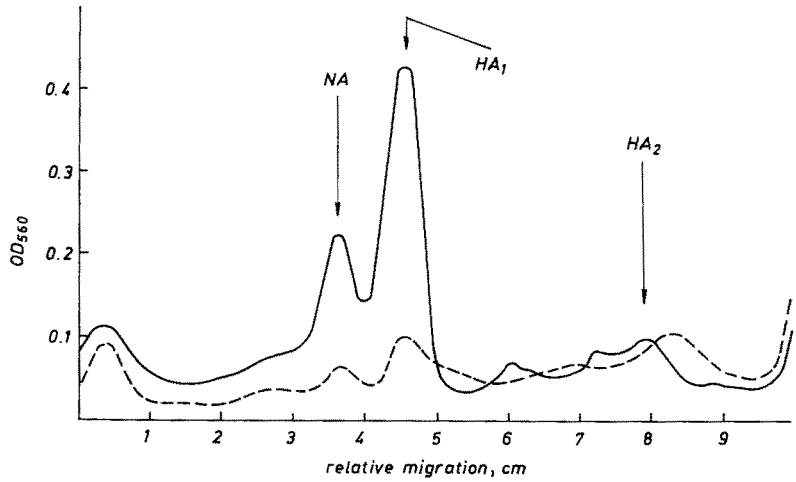

Fig. 3. The glycopeptide components of influenza virus MRC II after treatment with glycosidases. Virus was treated with enzymes as described in Materials and Methods. Untreated (———) and glycosidase-treated (— — —) viruses were electrophoresed on 10 per cent SDS-PAGE gel as described in the legend of Fig. 1. Following electrophoresis, the glycopeptides were visualized by staining with PAS reagent and then scanned. The resulted curves were redrawn after a base line correction. Electrophoresis was from left to right

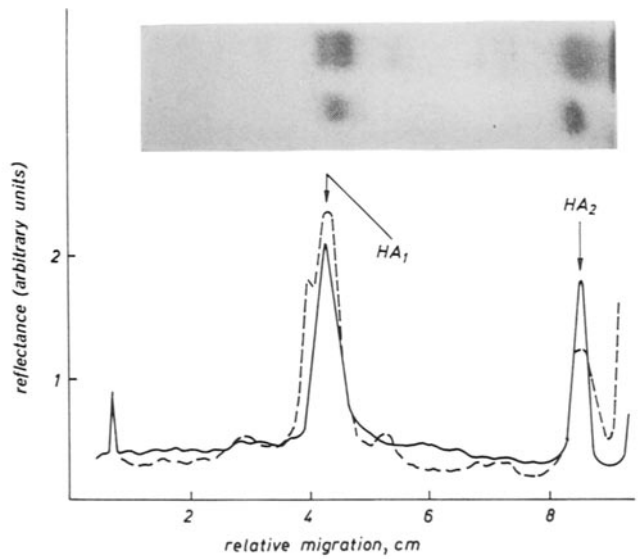

Fig. 4. Analysis of glycosidase-treated HA of influenza virus by electrophoresis in SDS-polyacrylamide gels. Purified HA was treated with glycosidase enzymes as described in Materials and Methods and electrophoresed on 10 per cent SDS-PAGE gel as described in the legend of Fig. 1. Gels were stained with amido-black and scanned. The resulting curves of untreated (———) and glycosidase-treated (— — —) HA were redrawn after a base line correction. The upper panel shows the amido-black stained gel of glycosidase-treated (top) and untreated (bottom) HA. Electrophoresis was from left to right

There was a correlation between these data and the amount of carbohydrate removed from intact virus and its purified HA after glycosidase treatment. The quantity of sugars released was determined by the ferricianide reducing test and are shown in Table 1.

Table 1. *Amount of carbohydrate removed from influenza virus and purified HA by glycosidase treatment*

Preparation	Amount of carbohydrate mMole/100 μg of protein	Carbohydrate content- per cent of total[a]	Percentage of sugar released[a]
1. Intact virus	23.5—30.4	4.5—5.5	
2. Glycosidase-treated virus	12.2—16.4	2.2—3.0	45 —52
3. Supernatant fluid after pelleting the treated virus	11.3—14.8	2.0—2.7	45.5—48.8
4 Isolated HA	82	14.8	
5. Glycosidase-treated isolated HA	48 —51.8	8.6—9.3	58.6—63.0

[a] Each value represents the mean of three experiments

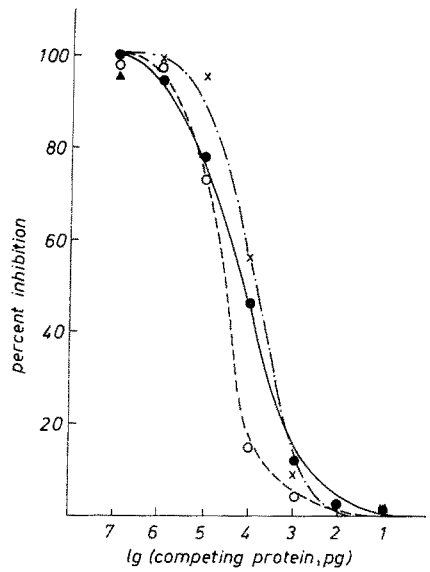

Fig. 5. Effect of glycosidase treatment on the antigenic reactivity of virus bound HA in competition radioimmunoassay. ^{125}I-labeled HA (40,000 cpm) and a limiting antibody dilution (1:2000) of anti HA serum were used in the reactions. Each point represents the mean ± range of duplicate determinations. MRC II was treated with glycosidase enzymes as described in Materials and Methods. Competing antigens were: (•———•) initial virus; (× — · — ×) control virus incubated under the same conditions but without glycosidase, (o — — — o) glycosidase-treated virus

Antigenic Reactivity of Virus Bound HA Treated with Glycosidases

The effect of the removal of carbohydrate by glycosidase digestion on the antigenicity of virus bound HA was determined by competition RIA. Hemagglutinating activity was measured in virus samples used as competing antigens 320,000 HAU/mg of protein were obtained in an untreated virus sample; 80,000 HAU/mg in a control virus sample (virus incubated under the same conditions but without glycosidase) and 640 HAU/mg in a glycosidase treated virus sample. The same samples were used for gel elctrophoresis or analyzed for the release of carbohydrate to ensure that the tests were made on the material from which carbohydrate had been cleaved.

Digestion of influenza virus with the mixture of four glycosidases had no effect on the antigenicity of the HA. Fig. 5 shows that the inhibition of binding of iodinated HA to its homologous antisera by glycosidase treated and untreated virus bound HA was the same. In both cases the same concentration (20 to 50 μg of protein) inhibited the binding of iodinated HA to its homologous antiserum by 50 per cent.

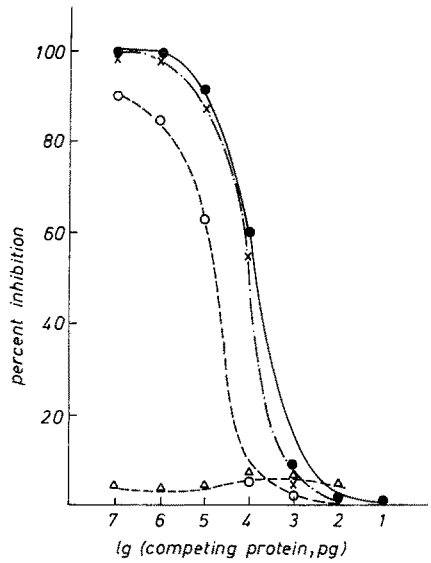

Fig. 6. Effect of glycosidase treatment on antigenicity of purified HA in competition radioimmunoassay. The reaction mixture was the same as described in the legend of Fig. 5. Each point represents the mean ± range of duplicate determinations. Competing antigens were initial HA (•———•), control HA incubated under the same conditions without glycosidase (× — · — ×) glycosidase-treated HA (○— — —○), glycosidase control (△— — —△)

Antigenic Reactivity of Purified HA Treated with Glycosidase

Isolated influenza virus HA was treated with a mixture of the four glycosidases and its antigenic activity was measured by competition assay. Fig. 6 shows that purified HA treated with glycosidase competed more than 8-fold less efficiently than intact purified HA. The amount of glycosidase-treated HA re-

quired for 50 per cent inhibition of the binding of ^{125}I-labeled HA to its homologous antibodies was more than 8-fold higher than intact HA. The incubation temperature and low pH in the control sample without glycosidase treatment had no effect on the antigenicity of purified HA. When a control HA sample was used as the competing antigen a competition curve identical to that of the initial sample of HA was obtained. The glycosidase control showed no reactivity at any concentration (Fig. 6).

The antigenic characteristics of HA were examined further by measuring the reactivity of ^{125}I-labeled HA in a direct RIA with anti-HA serum. The capacity of homologous antiserum to precipitate undigested and glycosidase-digested ^{125}I-labeled HA was examined, and the results are shown in Table 2. Glycosidase digestion of iodinated HA resulted in a decrease in the radioactivity precipitated with homologous antibodies over a wide range of antiserum concentrations (1/20 to 1/20,000). A control sample of iodinated HA (incubated without glycosidase) showed a slight decrease in the amount of radioactivity precipitated with anti-HA antibodies. Although incomplete glycosidase digestion of ^{125}I-labeled HA may account for these observations, the extent of the removal of the sugars could not be established with the small amount of iodinated HA available.

Table 2. *Amount of ^{125}I-labeled HA precipitated by homologous serum*

	Antiserum dilutions						
^{125}I-labeled HA	1/20	1/80	1/320	1/1,280	1/5,120	1/20,480	1/81,000
1. Initial sample	1.0[a]	0.95	0.93	0.57	0.27	0.12	0.03
2. Control sample[b]	0.94	0.79	0.52	0.22	0.17	0.06	0.02
3. Glycosidase treated sample	0.63	0.54	0.38	0.23	0.1	0.04	0

[a] The amount of ^{125}I-labeled HA precipitated by the homologous antiserum at 1/20th dilution was taken as 1.00. All values represent the mean of three determinations
[b] ^{125}I-labeled HA was incubated under the same conditions but without glycosidase

Discussion

A large part of the carbohydrate was removed from MRC II (H3N2) influenza virus and its isolated HA on treatment with the exoglycosidase enzyme mixture (α-mannosidase, β-N-acetylglycosaminidase, β-galactosidase and α-fucosidase). The glycosidase action was specific, with no effect on the integrity of the protein part of the molecule. We have shown that the glycosidase enzymes removed 50 per cent of the carbohydrate from the surface glycoproteins of intact virions, and 60 per cent from purified HA. Since slightly more carbohydrate was released from isolated HA than from the surface glycoproteins (HA + NA) of the virus, it is likely that the virion structure prevented the enzymes from acting on the oligosaccharide chains. The HA_2 carbohydrates on intact virus particles have been shown to be less accessible to glycosidases than those on purified soulble HA (8).

Influenza MRC II virus and its isolated bromelain-cleaved HA were shown to contain 4.5 to 5.5 per cent and 14.8 per cent carbohydrate, respectively. Similar values have been reported for H0 and H2 virions and their HAs (7, 13, 23, 26).

The effect of the removal of carbohydrate on the biological functions of HA was measured. A significant decrease in HA activity of the virus was seen after removal of the carbohydrate. The HA titer of glycosidase-treated virus was 0.2 per cent of the initial titre. These results are in agreement with our previous data (3) and other data on the inhibition of glycosylation of influenza proteins (32, 41). The stability of HA after glycosidase treatment of influenza H0 virus (8) may be due to the removal of only a small proportion of the carbohydrate.

Relatively little is known at present about the number, nature and structure of the antigenic determinants on influenza virus. Since HA_1 is exposed at the outer surface of the virion, it seems reasonable to expect that it contains the antigenic sites. Electron-micrographs of WRIGLEY *et al.* (52) indicated that all the anti-HA antibodies appeared to be attached near the distal end of the molecule, and that they were unaffected when HA was treated with detergents or isolated with proteolytic agents. It is known that HA_1 contains most of the HA carbohydrates (49, 36).

Further studies have shown that a mixture of peptides derived from HA by cyanogen bromide cleavage also bound specific antibodies (16). Furthermore, the antigenic activity of HA resided within the 170 N-terminal amino acid residues of HA_1 which also contained most of the carbohydrate (17).

It will be interesting to examine the possible role of carbohydrate in determining the antigenicity of HA glycoprotein.

No change in HA antigenicity of virus particles was observed after glycosidase treatment. However, there was a decrease in reactivity on treating purified HA with glycosidase. Glycosidase treatment produced an 8-fold difference in the amounts of control antigen and the glycosidase-treated antigen required to displace 50 per cent of ^{125}I-labeled HA. In addition, glycosidase digestion decreased the ability of homologous antiserum to precipitate ^{125}I counts in direct RIA. The difference in the decrease in antigenic reactivity of isolated HA and virus bound HA after removal of carbohydrate may depend on a higher conformational stability of the latter.

The decrease in antigenicity of glycosidase-treated isolated HA may imply that carbohydrate side chains are indirectly involved in the glycoprotein antigenic reactivity by the stabilization of the conformation of their antigenic determinants. Not all of the carbohydrate was removed by the glycosidase enzyme mixture, and the remainder might be important in maintaining the configuration of the molecule. Complete digestion of the carbohydrate side chains may produce a major change in the immunological reactivity of the glycoprotein. Antiserum, raised against fully glycosylated HA, appeared to react primarily with the polypeptide portions of the molecule. However the specificity of the reaction probably depended on the molecular conformation of HA, which could be at least partially influenced by the carbohydrate.

The role of carbohydrates in the immunological activity of viral glycoproteins had been studied for several oncoviruses (45, 37, 5) and the antigenicity of only

GP85 of avian myeloblastosis virus (45) has been reported to be influenced by the carbohydrate.

However, it can not be excluded that carbohydrates are responsible for determining the specificity of the antigenic determinants of the glycoprotein in a similar way to that described for the human blood group antigens (46) and the carcinoembryonic antigen of the human digestive system (43).

The lack of an appreciable difference between glycosidase-treated and untreated hemagglutinin in our experiments may be due to several factors, such as the extent of the removal of carbohydrate. On the other hand, oligosaccharide chains may stabilize large domains of the HA molecule, so that on removal of carbohydrate the efficiency of hemagglutination is reduced, whereas antigenicity is unaffected. However, our studies were done with a single influenza virus strain: an analysis of a wider range of virus strains should give more information on the role of the carbohydrate component on the immunological characteristics of influenza virus surface antigens. Such data would lead to a better understanding of the structure of influenza virus surface antigenic determinants and of the role of carbohydrate in the biological activity of these antigens.

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Authors' address: Dr. A. K. Gitelman, D. I. Ivanovsky Institute of Virology, U.S S.R. Academy of Medical Sciences, 16, Gamaleya str., Moscow 123098, U.S.S.R.

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